# ACID-SOLUBLE PHOSPHATE COMPOUNDS OF CORN ROOTS

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
December, 1962

ACKNOWLEDGEMENT

The writer expresses his appreciation to Dr. T. E. Humphreys for his thoughtful guidance during the course of this research; to Dr. G. R. Noggle for encouragement and financial support through the Department of Botany during the entire doctoral program; to Dr. A. T. Wallace for encouraging the writer to undertake the program; to Drs. R. D. Powell and T. W. Stearns for serving on the committee.

Special credit is due my wife, Winnie Sue Wise, who made this work possible.

## GLOSSARY

P: phosphorus

PP: pyrophosphate

IP: inorganic phosphorus

TP: total phosphorus

G3P: glyceraldehyde-3-phosphate

MPG: monophosphoglycerate

2PGA: 2-phosphoglycerate

3PGA: 3-phosphoglycerate

DPG: 2,3-diphosphoglycerate

R5P: ribose-5-phosphate

G1P: glucose-1-phosphate

G6P: glucose-6-phosphate

F1P: fructose-1-phosphate

F6P: fructose-6-phosphate

FDP: fructose-1,6-diphosphate

HMP: hexose monophosphate

HDP: hexose diphosphate

DPN or DPNH: oxidized or reduced diphosphopyridine nucleotide

TPN or TPNH: oxidized or reduced triphosphopyridine nucleotide

AMP, ADP, ATP: adenosine mono-, di-, triphosphates

CMP, CDP, CTP: cytidine mono-, di-, triphosphates

GMP, GDP, GTP: guanosine mono-, di-, triphosphates

UMP, UDP, UTP: uridine mono-, di-, triphosphates

IDP, ITP: inosine di-, triphosphates

UPPG: uridine diphosphate glucose

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## STATEMENT OF THE PROBLEM

The glycolytic sequence represents a crucial segment in the intermediary metabolism of the plant and animal cell. The operation of the glycolytic system can be expected to be influenced by the levels of each substrate and by the activity of each enzyme, in the series.

The presence in higher plants of most of the glycolytic intermediates is documented. However, very little is known about either the concentrations of these compounds, and their associated coenzymes, in normal plants, or their changes in relation to physiological changes. The reasons for this paucity of information can be traced to the severe technical difficulties encountered by various workers in applying quantitative analytical methods to plant materials, particularly the higher plants.

The object of this investigation is to develop methods to determine the levels of various glycolytic intermediates in plant tissue (etiolated corn seedling roots) using ion exchange column chromatography as the basic means of separation.

## REVIEW OF LITERATURE

The methods employed in this investigation are based on the separation of acid-soluble, phosphorus-containing compounds of plant tissue prior to their quantitative determination by various methods. The study involves a broad general survey of methods, including phosphorus assay, assay of sugar phosphates, assay of associated coenzymes, extraction methods, various types of ion exchange chromatography, and, finally, a review of the methods which have been used to study phosphorus compounds in plants. The following review is presented in the above sequence. It will be noted that most of the work in the field has been done with animal tissues. It will also be noted that there is some overlapping in presenting the pertinent articles. This occurs because it was thought that a review stressing methods would be more useful if the recent work involving ion exchange chromatography be presented with a modicum of detail.

Many methods have been described to assay phosphorus in biological material. Most procedures depend on the intense blue color which is produced when the phosphomolybdate complex is reduced by various agents. The color intensity is measured in a photoelectric colorimeter against a standard series of known phosphate concentrations.

One of the earliest methods (15) utilized hydroquinone for reduction; however, the color was evanescent. Fiske and SubbaRow

(30) discussed several factors affecting accurate phosphorus determinations. Variations of their method, involving reduction by aminonaphtholsulfonic acid in the presence of $H_2SO_4$, are still popular (13). The method is reportedly insensitive to a 30% variation in acid, molybdate, or reducing agent concentration. Berenblum and Chain (17) described a method in which the phosphomolybdic acid is reduced to the blue complex by shaking with a mixture of butyl alcohol and stannous chloride and separating the blue alcoholic layer. The method is said to be insensitive to ten-fold increases of the reagents. The Gomori (31) modification uses the photographic developer Elon (methyl-p-aminophenol sulfate) for reduction.

In general, IP* is determined by measurement of color production after 10 to 15 min of development; TP is similarly determined after wet-ashing the sample in $H_2SO_4$.

The estimation of IP in the presence of ATP with a minimum of molybdate-catalyzed hydrolysis of ATP is described by Marsh (54). The excess molybdate is removed as a citrate complex after the extraction of phosphomolybdate by butanol. Alternately, the IP can be precipitated by $CaCl_2$-$Ca(OH)_2$ to remove various labile phosphate esters, such as creatine phosphate and ATP.

Several colorimetric methods used for estimation of sugars and sugar phosphates are described by Ashwell (10). The anthrone, cysteine-carbazole, and orcinol procedures (10) are colorimetric assays for hexose, fructose, pentose, and their phosphate derivatives. Two compounds as similar as ribose-3-phosphate and R5P may be differentiated under carefully controlled heating conditions (7). In

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* See glossary.

practice, the results have been equivocal (10) except where considerable purification was attained prior to colorimetry. However, Bartlett (13) has reported the determination of ketohexose phosphate with cysteine-carbazole, and pentose phosphate with orcinol, in fractions containing a mixture of these esters. Further, Bartlett has claimed quantitative assay of G6P by subtracting the cysteine-carbazole result from the anthrone result obtained on assaying a mixture of G6P and F6P. However, Helbert and Brown (35) found that color production in the anthrone assay varied from hexose to hexose. G1P has been estimated in mixtures of hexose phosphates by measuring the increase in IP after 7 min at 100 C in 1 N HCl (45).

Enzymatic methods are available for the estimation of sugar phosphates. FDP can be assayed by the method of Slater (70), using the rabbit muscle fraction A of Racker (63), which contains aldolase, triosephosphate isomerase and α-glycerol phosphate dehydrogenase. The change in optical density at 340 mμ due to oxidation of added DPNH is used to calculate FDP. The method measures the sum of FDP, G3P, and DHAP.

F6P can also be estimated by Slater's procedure (70). When Racker's (63) rabbit muscle fraction B (containing phosphohexokinase) and ATP are incorporated in the above reaction, FDP and F6P are measured. F6P is measured by the difference between this method and that for FDP alone, in the absence of G6P.

G6P can be assayed by the method of Ochoa et al. (60) by measuring the change in OD due to reduction of TPN by glucose-6-phosphate dehydrogenase. If phosphohexose isomerase is added to the system, F6P may be estimated by difference (70). Greengard et al. (33) estimated G6P,

according to the procedure of Slater (70), by measuring the change of fluorescence of TPNH, and, alternatively, measured ATP by adding glucose and hexokinase to the reaction mixture used for the estimation of G6P. Kornberg (40) determined ATP by this method (spectrophotometrically). Ochoa et al. (60) assayed pyruvate spectrophotometrically in the presence of lactic dehydrogenase and DPNH.

Seraydarian et al. (69) adapted fluorometric methods to the determination in muscle extracts of millimicromolar quantities of (a) FDP plus triose phosphates and (b) hexose monophosphates. The two groups were not separated; the authors felt that the two groups were important in representing glycolytic intermediates of similar phosphate composition.

The levels of adenine nucleotides, especially ATP, are particularly important in biochemistry of animal and plant tissues. ATP has been estimated by the method of Slater (70) and the micro method of Seraydarian (69). McElroy's finding (47) that added ATP caused light production in the darkened extracts of firefly lanterns opened the possibility of using the phenomenon to assay ATP. Further experiments by the McElroy group (48) showed that the production of light in firefly extracts (FFE) depends on a heat-labile enzyme (luciferase), a heat-stable, yellow-green luminescent compound (luciferin), an inorganic ion ($Mg^{++}$, $Co^{++}$, or $Mn^{++}$), oxygen, and ATP. A partial purification of the FFE (49) resulted in the removal of myokinase and adenosinetriphosphatase, and revealed the specificity of the luciferin-luciferase reaction to ATP. Strehler and Totter (71) described the application of the system to the estimation of mixtures of ADP and ATP.

The FFE assay for ATP has been used in investigations on animal tissues, especially the nervous system. Single frog sciatic nerves (ca. 25 mg) were heated in boiling 'Tris' buffer for 15 sec, chilled in ice, homogenized in the cold, shaken with $CCl_4$, and centrifuged. ATP was estimated on the clear supernatant, using controls also containing $CCl_4$ (33). Weiner (73) employed special procedures to minimize the rapid breakdown of labile phosphate compounds which occurs in the central nervous system of rats killed by decapitation. The animals were frozen rapidly by immersion in liquid oxygen, the brain was chipped out and extracted in cold perchloric acid, and the acid was precipitated with KOH. The change in optical density at 251 and 265 mu upon addition of adenylic acid deaminase was assumed to be equivalent to AMP in the sample. Myokinase was then added, and the change in $OD_{265}$ was proportional to one half the amount of ADP in the sample. Blanks and known mixtures of nucleotides were run with each series, and corrections were made for slight non-specific change in $OD_{251}$, due to addition of enzymes. Cheng (22), working with trichloroacetic acid extracts and perchloric acid extracts (1) of nerve trunks, coupled the following enzyme systems, each followed by a heat inactivation step: (a) Pyrophosphorolysis of ATP in presence of sulfurylase (64) and pyrophosphatase (41) (b) Phosphorylation of ADP to ATP with creatine phosphokinase (42) (c) The final assay of ATP (phosphorylated ADP) with luciferase (71).

Minard and Davis (58) used even more exacting methods of extraction of rat brains than did Weiner (73) for the separation of nucleotides, especially ATP. They immersed the rat suddenly in

liquid nitrogen for several minutes. The brain was chipped out, collected in a mixture of Dry-Ice and acetone, quickly pulverized, and extracted in trichloroacetic acid at 0 C. Chromatographic separation of nucleotides was based on the method of Hurlbert et al. (38). Phosphorus measurements and $OD_{260}$ readings were made on each fraction to calculate the total nucleotide concentration. There was no correction for formate, which absorbs strongly in the ultraviolet region. The positions of adenine, guanine, and uridine nucleotides were determined by comparison with chromatograms of known nucleotides. The peak fractions were pooled, subjected to hydrolysis in 1 N HCl, and the individual bases were identified by paper chromatography. Portions of the eluate fractions containing AMP, ADP, and ATP were passed through columns of Dowex 50 resin (hydrogen form) to remove ammonium ion, evaporated to dryness in vacuo to remove formic acid, and hydrolyzed. Components of the hydrolyzates were separated with a Dowex 1-chloride column developed with 2-amino-2-methyl-1,3-propanediol and HCl. This system can separate adenine, guanine, CMP, UMP, and AMP.

Bishop et al. (19) separated the nucleotides in trichloroacetic acid extracts of whole blood, according to Hurlbert et al. (38); but non-gradient elution was used. The material in each peak was evaporated to dryness on a steam bath, and the free purines and pyrimidines liberated by hydrolysis for 1 hour at 72 C in 72% perchloric acid. Neutralized solutions were chromatographed in the isopropanol-HCl system of Wyatt (74) followed by the butanol-$NH_4OH$ system of Markham and Smith (51). The bases were eluted with dilute HCl, and their spectra determined.

Cohn (23) introduced ion exchange column chromatography into the

nucleotide field. Adenylic, uridylic, guanylic and cytidylic acids were eluted from Dowex 1-chloride polystyrene anion exchange resin columns by dilute HCl-NaCl solutions in a sequence roughly predictable from pK values. Chromatograms were prepared by measuring $OD_{260}$ of each fraction collected and plotting these values against the volume eluted. Cohn and Carter (24) similarly eluted AMP, ADP, and ATP. Paper chromatograms were developed on material from peak tubes, using an isoamyl alcohol-disodium phosphate mixture and the nucleotides detected by ultraviolet fluorescence.

Horecker and Smyrniotis (36) reported a partial separation of R5P and a similar substance later found to be ribulose-5-phosphate on a Dowex 1-formate column by elution with 0.1 M formate buffer. This was a pioneer experiment in the elucidation of the pentose phosphate shunt. The pentose phosphates were produced when a purified yeast enzyme was mixed with 6-phosphogluconate.

Benson et al. (16) separated F6P from FDP on a Dowex 1-chloride column eluted with 0.1 M NaCl. Khym and Cohn (39) reported the separation of synthetic mixtures of sugar monophosphates (G1P, G6P, F6P,R5P) by forming borate complexes simultaneously with elution from Dowex 1-chloride column with four chloride solutions. Hexose phosphates were estimated by the anthrone method (59), R5P by the orcinol method (21), and phosphorus was assayed according to Fiske and SubbaRow (30).

Goodman, Benson and Calvin (32) used the above techniques of Khym and Cohn (39), Benson et al. (16), and Horecker and Smyrniotis (36) to study photosynthetic products in ethanol extracts of the alga, Scenedesmus. A synthetic test mixture of F6P, FDP, and 3PGA, and a

mixture of 3PGA, FDP, and Ribulose-1, 5-diphosphate was separated on Dowex 1-chloride by elution with a dilute NaCl-HCl solution. They also separated F1P, F6P, and G6P by elution with 0.1 M $Na_2B_4O_7$ from a column converted to the borate form by eluting a Dowex 1-chloride column with $Na_2B_4O_7$. Finally, they chromatographed a simple synthetic mixture of the above sugar phosphates mixed with an extract from algae grown in the presence of $P^{32}$. Radioactivity was found in peaks corresponding to various esters, indicating the natural incorporation of phosphorus in those compounds by the algae.

Aisenberg (2) used Dowex 1-chloride columns mainly to remove free sugars from the glycolytic intermediates in the acid-soluble fraction of high-speed supernatant of brain extract. The free sugars were washed through the column with water, the esters were eluted batchwise with 0.1 N HCl, the eluate was concentrated by lyophilization, and G6P, F6P, G3P and hexose diphosphate were enzymatically assayed (25).

Bergkvist and Deutsch (18) separated synthetic mixtures of mono-, di- and triphosphates of adenine, guanine, and uridine by applying seven successively increasing concentrations of formate buffer. $OD_{260}$ of each fraction was plotted against volume through the column and identification of each peak was made by comparing its UV spectrum with spectra of known nucleotides.

Diedrich and Anderson (28) used the method of Goodman et al. (32) to separate galactose-1-phosphate from other common hexose monophosphates in a synthetic mixture. They described two methods to remove the troublesome borate ion from column eluates. They found, as had Helbert and Brown (35), that the color in the anthrone assay (65)

varied from hexose to hexose, so they computed values from each parent sugar. The method they generally used to monitor fractions was based on Dische et al. (29). This modification enabled them to distinguish pentose from hexose in mixtures.

Diedrich and Anderson (27) used similar methods in studying the appearance of galactose. Trichloroacetic acid extracts were resolved into several anthrone-positive peaks, each of which was revealed by paper chromatography to contain several unidentified compounds. The fractions of the first two peaks were pooled, and tested enzymatically for galactose-1-phosphate.

Tiselius et al. (9) discussed some limitations of stepwise elution and introduced a new general procedure for "gradient elution" of mixtures of carbohydrates. Gradient elution has since been successfully applied to mixtures of organic acids, amino acids, peptides, proteins, and nucleotides. Lakshmanan and Lieberman (43) stated the advantages of using concave gradient elution, in which the rate of change of eluent concentration increases with the concentration. The more easily eluted substances are spread apart in the chromatogram, and, since the concentration toward the end of the chromatogram increases rapidly, the more tightly bound substances are eluted without undue delay. This system also produces narrow peaks with a minimum of tailing. Pontis and Blumson (62) used concave gradient elution to separate a synthetic mixture of nucleotides on an ion exchange resin. Bock and Nan-Sing Ling (20) described systems generating several types of elution gradients.

Hurlbert et al. (38) described a system of "extended gradient elution." The reservoir, containing the concentrated eluent, and

the mixing vessel, initially containing water, were designed so that the contents of the reservoir could be changed at intervals. Several ammonium formate buffers were used to separate the nucleotides in synthetic mixtures. The nucleotides in a perchloric acid extract of rat liver were similarly separated. Martonosi (55) used extended gradient elution (38), a Dowex 1-column (bicarbonate form), and $KHCO_3$ eluent solutions to separate a synthetic mixture containing AMP, ADP, ATP, DPN, IP, TPN, PP, IDP, and ITP. Bicarbonate was removed from eluate fractions by neutralizing with perchloric acid, followed by centrifugation of the potassium perchlorate precipitate. Adenine and adenine nucleotides were assayed from $OD_{260}$ values; DPN, IP, TPN, PP, IDP, and ITP were assayed by IP and TP determination.

Mills (57) used extended gradient formate chromatography to separate several nucleotides in red blood cells. Monitoring was done by measuring $OD_{260}$ of each fraction. Numerous chromatograms of known nucleotides were run for comparison. Several nucleotides were quantitatively estimated by using the appropriate extinction coefficients after they were first identified by comparing their absorbancy ratios in the region from 255 to 290 mu with the ratios of known nucleotides and derivatives. (61)

Wade (72) devised a complicated system, employing pumps which operated automatically in response to a pH-sensing device, to separate various synthetic mixtures of phosphate esters. The system was designed to change the pH over a much wider range than could be obtained by changing buffer concentration.

Bartlett (12) reported a very extensive investigation of glycolytic intermediates and co-enzymes in red blood cells. This work

was based on anion exchange chromatography, but many related techniques were employed.

Studies on phosphorylated compounds in plants to the year 1952 are reviewed by Albaum (4). In the earliest studies, the acid-soluble fraction was assayed for inorganic phosphorus and organic phosphorus. ATP was measured by determining the increase in IP after hydrolyzing the acid extracts for 7 minutes at 100 C in 1 N HCl; it was assumed that all phosphorus labile under these conditions is ATP phosphorus.

In 1943 LePage and Umbreit (49) presented a system to determine specific P-compounds in synthetic mixtures of pure compounds. This method was based on the finding that IP, ATP, ADP, PGA, and FDP form soluble barium salts under the same conditions. They were able to apply this method to trichloroacetic acid extracts of Thiobacillus thioxidans by carefully controlling the quantity of tissue and reagents. IP was determined directly. PGA was estimated by a colorimetric reaction then thought to be specific. FDP and F6P were estimated by a colorimetric method. G1P was estimated as "seven minute phosphorus." DPN was estimated from the concentration of nicotinamide. AMP was estimated from nitrogen and ribose determinations, after correcting for DPN. ATP and ADP were measured by determining total nitrogen as an index of purine present, pentose assay, and ratio of labile phosphorus to total organic phosphorus, after deducting the phosphorus resulting from other compounds present. Correction had to be made for phosphorus hydrolyzed from FDP (about 27%). The remaining phosphorus was assumed to be G6P. Despite the large number of assumptions and corrections made by LePage and

Umbreit, they reportedly accounted for over 90% of the acid-soluble phosphorus.

Since this general method worked in bacteria (and animal tissues), Albaum and Umbreit ( 8 ) attempted to apply it to higher plants (oat seedlings). Large amounts of non-specific absorption resulting from the presence of various polysaccharides, frustrated theri efforts to apply colorimetric sugar assays. Also, after applying all the corrections, much phosphorus still could not be accounted for. It was known that cereals contain quantities of phytin but correction for phytin still left some phosphorus unaccounted for.

Albaum and Ogur ( 5 ) tried to isolate ATP from oats by traditional methods of fractionation with barium and mercury. Although polysaccharide could be removed from animal tissues by precipitation with ethanol, this was not true of the oat extracts; polysaccharides were carried along in each precipitate and each supernatant. Also, barium phytate was precipitated under almost the same conditions as ATP. Finally, there was much unknown material absorbing nonspecifically in those parts of the ultraviolet spectrum where the absorption of nucleotides was maximum. Albaum et al. solved the problem neatly by turning to a non-cereal source of plant material, the mung bean. This material yielded ATP of about 70% purity. The Albaum group ( 6 ) also tried to apply the fractionation to Euglena. The presence of inorganic phosphate, pyrophosphate, metaphosphate, AMP, ADP, ATP, DPN, G1P, F6P, PGA, and riboflavin phosphate was reported. Marre et al.(53) studied the effects of auxin treatment and pollination on levels of ATP and some glycolytic intermediates in tomato ovaries, by the methods of LePage and Umbreit (46).

Most of the early fractionation procedures required large amounts of plant material. In the isolation of ATP from mung beans 5 to 10 pounds were used. Later column chromatography and paper chromatography were used with smaller samples of material. Albaum (4), working with mung bean seedlings, prepared trichloroacetic acid extracts from 3 g of tissue, precipitated the P compounds with ethanol and barium, and precipitated barium with $H_2SO_4$. The solution was poured through a small column of Dowex 1-chloride, and eluted stepwise with five increasingly concentrated chloride solutions, displacing adenosine, adenine, AMP, ADP, and ATP in succession.

Laughman and Martin (44) separated acid-soluble organic phosphorus compounds from roots after short periods of absorption of $P^{32}$ by young barley plants. Extraction by 0.2 N HCl, 0.5 N trichloroacetic acid, or 80% ethanol gave similar results. Separation was performed by paper chromatography by the method of Hanes and Isherwood (34). Five radioactive spots were resolved by a butanol-water-picric acid system. Each had the appearance of a single compound, but each was composed of two or more substances, which could be separated by the use of other solvent systems. Unknown radioactive compounds were mixed with the pure unlabelled forms of suspected compounds. Chromatograms were then run in solvent systems separating the known substance from others. Active areas were compared with those developed by a molybdate spray, revealed under UV light.

Several investigations have dealt with the assay of phosphate esters in the fruit and seedling of pea, Pisum sativum L. Rowan et al. (68) reported changes in levels of ADP, ATP, HMP, and HDP in perchloric acid extracts of pea fruits at various stages of maturation, by

Slater's procedure (70). There was a possibility of interference in the determination of ATP by UTP and GTP (67), but, since paper chromatograms of the nucleotides showed the latter materials to be present in small amounts, no correction was made. Rowan (66) extended the investigation to the quantitative determination of uridine mono-, di-, and triphosphates, as well as ADP and ATP. In this work a large amount of material was used (250 g to 2 kg).

Marre and Forti (52) extracted stem sections of seven-day-old pea seedlings in perchloric acid. The extract was treated according to the method of Crane and Lipmann (26) with "Norite A" charcoal, which absorbs nucleotides but not sugar phosphates. The nucleotide-containing Norite A was filtered, heated in a boiling water bath for 10 minutes in 1 N HCl, and IP was assayed. Crane and Lipmann (26) extended this procedure to measure ADP and ATP labile phosphorus, but other nucleotides might be present in such an extract, which would result in high readings.

## MATERIALS AND METHODS

### Plant Materials

Dried kernels of hybrid seed corn (Zea mays L., var. Funk's G-50 and G-740) were rinsed thoroughly, and soaked with aeration in tap water for 24 hr. The kernels were rinsed again and then placed individually on trays lined with thoroughly wetted filter paper. Each tray was covered and the kernels were allowed to germinate in the dark at 25 C for 72 hr. The distal 2 cm was cut from the primary root of each seedling and collected in ice-cold distilled water. The root tips were filtered in a Buchner funnel, washed once with ice-cold water, gently spread on filter paper to remove adhering water, and weighed to the nearest 0.1 g. A typical harvest of 650 to 700 root tips yielded about 20 g of tissue.

### Preparation of Extracts

The weighed root tips were thoroughly ground for about 3 min in 10% trichloroacetic acid (1 ml/g of tissue) in an ice-cold mortar. The resulting slurry was centrifuged, and the supernatant liquid was decanted. The residue was suspended in 5% trichloroacetic acid (1 ml/g) and centrifuged. The combined supernatant fractions were retained; the residue was discarded. All manipulations to this point were performed at 0-5 C. The trichloroacetic acid was removed by four extractions with two volumes of cold ether; the pH of the extract was then about 3.5. A thin stream of nitrogen was bubbled through the

extract until the ethereal odor was not discernible, and 5 N $NH_4OH$ was added to pH 6.8-7.0. This extract was passed through a Dowex 50 column, and followed by 50 ml of water; the combined filtrate was neutralized to pH 6.8-7.0. One to four extracts were used in chromatography. The extracts could be stored several weeks at -20 C.

Preparation of Ion Exchange Columns

Trimethylammonium polystyrene ("Dowex") resins (Dow Chemical Co.) were obtained in a purified form (Bio-Rad Laboratories, Richmond, Calif.). Two types of anion exchange resins and one type of cation exchange resin were used.

One anion exchange resin, AG 1-X8, 50-100 mesh, was suspended in water and the extremely fine particles were decanted. Fourteen-cm resin beds were formed in 1 X 30-cm glass chromatographic tubes with sintered glass retainers (Emil Greiner Co.). Uniformity of the many columns prepared during these investigations was approached by stirring thoroughly the thin resin slurry immediately before pouring the column, and repeatedly inverting the column while adjusting the resin bed to the desired length. The resin columns were each capped by a small plug of washed glass wool, and eluted successively with two bed volumes of 88% formic acid (38), three volumes of water, three volumes of 1 N $NH_4Cl$, and, finally, with water until the filtrate had the same pH (5.5-6.0) as the distilled water used, and was also chloride-free. Chromatographic columns prepared in this manner are subsequently referred to as "Dowex 1-chloride" columns.

The other anion exchange columns, containing AG 1-X8, 200-400 mesh, were prepared as follows. The extreme fines were decanted and the amorphous particles were removed with a medicine dropper. One X

14-cm resin beds were poured as described above and eluted with three bed volumes of 88% formic acid, four volumes of 5 N ammonium formate (until the filtrate was chloride-free), and with an excess of water. Such resin columns are referred to herein as "Dowex 1-formate" columns.

The cation exchange columns were prepared from AG 50W-X8, 100-200 mesh. Three-cm beds were poured in 1 X 30-cm tubes. The resin was eluted with 30 ml of 2 N HCl, with water until the filtrate was chloride-free and with an excess of water.

Column Chromatography

Solutions containing anions to be separated by ion exchange column chromatography were passed through Dowex 1-formate columns at flow rates of about 3.0 ml/min. Solvent flow was maintained by two to four pounds of air pressure from a low pressure regulator (Matheson Co.) and adjusted by a teflon-glass needle valve (Emil Greiner Co.). Connections were made with silicone rubber tubing Type HTR (Ronsil Co., Little Falls, N.J.) after it was found that "tygon" tubing introduced significant contamination in colorimetric analyses of the eluate fractions. The fractions were collected by an automatic fraction collector (Research Specialties Co.), with a volumetric siphon; graduated test tubes were used until the reliability of a siphon was established. Twenty-ml fractions were collected from Dowex 1-chloride columns and 10-ml fractions were collected from Dowex 1-formate columns. Each fraction was assayed for TP, and chromatograms were always prepared to reveal the elution positions of P-containing compounds.

The anions that were adsorbed on Dowex 1-chloride columns were eluted at about 2.8 ml/min by 200-400 ml of each of the following

solutions (13): 0.01 N HCl, 0.02 N HCl; 0.1 N $NH_4Cl$; 0.2 N $NH_4Cl$, 0.5 N $NH_4Cl$.

In preliminary experiments, the fractions obtained with each of the five chloride eluent solutions from a Dowex 1-chloride column, containing compounds of similar resin-binding capacity, were pooled, passed through a Dowex 1-formate column, and the anions were eluted by formic acid or ammonium formate buffers. In later experiments the Dowex 1-chloride column eluate was collected in five batches corresponding to the five eluent solutions. Each batch was neutralized with $NH_4OH$ to pH 6.8-7.0, and was stored at -20 C until chromatographed with a Dowex 1-formate column.

Dowex 1-formate columns were also used to chromatograph directly whole trichloroacetic acid root tip extracts without prior separation on Dowex 1-chloride columns. The fractions in a TP peak were often pooled, passed through Dowex 50 (hydrogen form) to remove ammonium ion, extracted several times with ether to remove formic acid, neutralized, and retained for analysis.

Dowex 1-formate columns were eluted at about 1.0 ml/min with linear or concave gradients of formic acid or ammonium formate buffer. Concentration gradients were obtained with systems diagrammed by Bock and Ling (20). A linear gradient was obtained when the cross-sectional areas of the two eluent-containing vessels were the same. Concave gradients were obtained when the area ($A_1$) of the mixing vessel was greater than that of the reservoir ($A_2$). The ratio, $\frac{A_2}{A_1} = 0.6$, was selected for use in these experiments (62). However, linear gradients were used more often than concave gradients after preliminary experiments with the latter.

Various synthetic test mixtures of known phosphate compounds were also chromatographed by the same procedure with Dowex 1-chloride or Dowex 1-formate systems for comparison with chromatograms of plant extracts.

Analytical Methods

Phosphorus (TP, IP, 7-min P) assay. Phosphorus was assayed colorimetrically by measuring the blue phosphomolybdate complex. The following modification of the Gomori (31) method permitted reliable and relatively convenient assay of large numbers of samples.

For TP assay, the sample was wet-ashed by heating for 3 hr in an oven at 170-175 C in a 10 X 180-mm test tube calibrated at 10 ml and containing 1.0 ml of 5 N $H_2SO_4$ and a glass bead. Two drops of 30% hydrogen peroxide were added, and heating was continued for an additional 2 hr. Three drops of 5% urea were then added and heating continued at 100 C for 3 hr. One ml of water was added and heating continued at 120-130 C for 1 hr. After the tube had cooled, two ml of 6.25% $Na_2MoO_4 \cdot 2H_2O$ in 5 N $H_2SO_4$ were added, followed by 1.0 ml of 1% Elon (31) (Eastman Co.) in 3% $NaHSO_3$. The color formed was read, after 10 to 60 min development, in a photoelectric colorimeter (Klett Co.) using filter #64. Calibration curves were prepared using an inorganic phosphate standard solution.

IP was assayed similarly, but without heating. The temperature was kept below 22 C, and the sample was diluted before addition of acid, to minimize hydrolysis of labile phosphorus compounds (27).

Acid-labile, or "7-min" phosphorus was estimated by the increase in IP after 7 min at 100 C in 1 N HCl.

Assays for sugar phosphates. Hexose was assayed by a modification

of the anthrone method (10). Two-ml samples were layered over 4.0 ml of 0.2% anthrone in 95% $H_2SO_4$ while chilled in an ice bath. The mixture was shaken vigorously, heated for 15 min at 100 C, and cooled in tap water; absorbancy was read in a colorimeter (Klett Co.; filter 60) against a standard calibration curve prepared from glucose or fructose. The mixture was usually shaken once during the heating step to remove bubbles.

Fructose was assayed as follows (10). To 2.0 ml of the sample were added 0.2 ml of 1.5% cysteine, 4.0 ml of 95% $H_2SO_4$, with cooling in an ice bath, and 0.2 ml of 0.1% carbazole in absolute alcohol. The mixture was shaken, heated at 60 C for 30 min, and cooled in tap water; absorbancy was read in a colorimeter (Klett Co.; filter 56), or in a Beckman DU spectrophotometer at 470, 560, 650, and 750 mu (10) against a standard calibration curve prepared from fructose.

Pentose was determined by the following modification of the orcinol method (10). To 3.0 ml of the sample were added 3.0 ml of 0.1% $FeCl_3$ in concentrated HCl (with cooling in an ice bath), followed by 3 drops of orcinol reagent (500 mg/ml of absolute alcohol). The mixture was shaken and heated for 10 to 20 min at 100 C. Absorbance was read in a photoelectric colorimeter (Klett Co.; filter 64) against standard curves prepared from arabinose, ribose, or R5P.

Assay for glycerates. Glycerates were analyzed as follows (14). The sample, in a volume of 0.2 ml, was added to 5.8 ml of 0.01% 4,5-dihydroxy-2,7-naphthalene-disulfonic acid (chromotropic acid). The mixture was shaken and then heated for 30 min at 100 C, cooled, and the absorbancy read in a colorimeter (Klett Co.; filter 69) or Beckman

DU spectrophotometer at 690 mu against a known standard solution.

Enzymatic assays for phosphorus compounds. An enzyme, or a mixture of enzymes, was used to assay G6P, F6P, and UDPG. In each case, the enzyme activity was tested against known substrate preparations. The change in optical density was measured by a Beckman model DU spectrophotometer using a quartz cuvette having a 1-cm light path. The level of substrate in a sample was computed from the molar absorbancy value of the reduced pyridine nucleotides at 340 mu ($6.22 \times 10^{-3}$), and this value was multiplied by the appropriate factor to give the results reported herein. A method for the determination of millimicromolar amounts of ATP is described in more detail.

G6P and F6P. G6P was assayed by following the reduction of TPN in the presence of glucose-6-phosphate dehydrogenase (G6PD) (37).

$$\text{G6P+TPN} \xrightarrow{\text{G6PD}} \text{6-phosphogluconate+TPNH+H}^{+}$$

The amount of G6P was calculated from the increase in $OD_{340}$ according to the expression

$$\text{G6P (umoles)} = \frac{(\text{OD})(3.0)}{6.22}$$

A typical reaction mixture contained 1.0 ml of 0.04 M glycyl glycine buffer (pH 7.5), 1.0 ml of 0.02 M $MgCl_2$, 0.2 ml of TPN (4 mg/ml), 5 ul of G6PD (Sigma Type V), and G6P, in a total volume of 3.0 ml.

F6P was assayed in the same way in the presence of G6PD and phosphohexose isomerase (50).

$$\text{F6P} \xrightarrow{\text{PHI}} \text{G6P}$$

Uridine diphosphate glucose (UDPG). UDPG was measured by

following the reduction of DPN in the presence of uridine diphosphate glucose dehydrogenase (UDPGD), which is specific for the reaction

$$\text{UDPG} + 2\text{DPN} \xrightarrow{\text{UDPGD}} \text{UDP-glucuronate} + 2\text{DPNH}$$

UDPGD was prepared from calf liver, according to Maxwell et al. (56) The amount of UDPG was calculated from the increase in $OD_{340}$ according to the expression

$$\text{UDPG (umoles)} = \frac{(\text{OD})(3.0)}{12.0}$$

A typical reaction mixture contained 0.06 ml of DPN (35 mg/ml, 0.3 ml of 1 M glycine buffer, pH 8.7, 0.5 ml of UDPGD, the sample containing UDPG, and water to a final vol of 3.0 ml.

Microdetermination of ATP with firefly lantern extract. The intensity of the luminescence is proportional to the ATP concentration (48). As little as $2 \times 10^{-4}$ umoles of ATP can be measured with a Farrand photofluorometer, using an extract prepared from firefly lanterns.

The extracts were prepared by the following modification of the method of McElroy (48). Fifty mg of dehydrated firefly lanterns (Sigma Chemical Co.) were ground in a small glass homogenizer (Kontes Glass Co.) for 3 min in 1-1.5 ml of 0.1 M $Na_2AsO_4$ buffer, pH 7.4, at 1-4 C. The extract was transferred to a graduated test tube. The homogenizer was rinsed twice with cold buffer, the rinsings and extract were combined, and the volume was made up to 5.0 ml with buffer. Cell debris was sedimented by centrifugation and discarded. Fifty mg of $MgSO_4 \cdot 7H_2O$ were dissolved in the supernatant liquid. This

preparation is herein referred to as firefly lantern extract (FFE). All manipulations were done in the cold. Filtration of the homogenate (71) was not as convenient as centrifugation, and activity of the filtered preparation was about 50% lower. The FFE retained 90% of full activity for 1 week at 5 C. A typical reaction mixture contained 0.2 ml of FFE, the sample to be assayed, and water in a total volume of 0.8 ml. Each preparation was calibrated against known ATP samples. The galvanometer was read 30 sec after adding the sample. Full scale deflection (10.0 units) was usually produced by 0.2 mumoles of ATP.

The presence of transphosphorylases in the FFE was confirmed by several experiments in which GTP or GTP plus ADP were added instead of ATP (11).

Trichloroacetic acid extracts of corn root tips were assayed in the following manner. A portion of the extract was diluted with water to a suitable concentration, and a 0.2 ml sample was assayed as decribed above. The galvanometer reading was multiplied by an appropriate factor to determine the total ATP in a known weight of plant tissue.

Single root tips were assayed as follows. A weighed, 2.0 cm root tip (25-30 mg) was homogenized in 1.0-1.5 ml of 1.5 M perchloric acid for 30 sec at 1-4 C. The homogenate was transferred to a small centrifuge tube, and the homogenizer was rinsed three times with about 0.5 ml of cold water. The pH was adjusted to 7.2 with KOH (1.5 M and 0.15 M), and the extract was centrifuged. The supernatant fluid was transferred to a graduated test tube; the precipitate was suspended in cold water and centrifuged. The supernatant fractions

were combined, and made up to 5.0 ml with water. All manipulations were done in the cold. A 0.1 to 2-ml portion of this extract was assayed with FFE.

Analysis from UV absorbancy measurements. Absorbancy was measured at 260 and 290 mμ on the fractions eluted from Dowex 1 columns. Wherever the ratio 260/290 was high, the presence of a nucleotide was suspected, and measurements at additional wavelengths were taken to characterize the nucleotide (61).

## RESULTS

### Dowex 1-Chloride Chromatography of Known Compounds

Test solutions containing mixtures of known P compounds were chromatographed with Dowex 1-chloride columns. The elution sequences were similar to those previously reported (13): AMP, G6P, F6P, and IP were eluted by 0.01 N HCl; ADP was eluted by 0.02 N HCl; FDP was eluted by 0.1 N $NH_4Cl$; ATP was eluted by 0.5 N $NH_4Cl$. The positions of the compounds were determined from chromatograms drawn from TP assay and $OD_{260}$ measurements on each fraction. Another Dowex 1-chloride column was eluted with the five solvent systems. Each fraction of the "blank run" gave a zero response to TP assay and had negligible absorbance at 260 mμ.

The anthrone and cysteine-carbazole assay methods were applied to the P-containing fractions. Some of the fractions showed surprisingly high absorbancy readings, due to the formation of bubbles. This result was most severe in the fractions containing the highest concentrations of ammonium ion, but could be minimized by thorough shaking of the test tubes with a mechanical test tube shaker several minutes before absorbancy measurements were made.

### Dowex 1-Formate Chromatography of Known Compounds

A "blank run," using 0 to 1 N formic acid, showed each fraction to be negative for TP. Absorbancy increased greatly with formate concentration in the 200-240 mμ range, but was negligible in the near

UV range. Anthrone and cysteine-carbazole assays on the same fractions gave low but erratic readings due to bubble formation in the presence of formate ion; vigorous shaking was necessary to minimize errors from this source.

A test solution containing AMP, G1P, G6P, F6P, R5P, and IP was chromatographed. The anthrone, cysteine-carbazole, and orcinol assay methods were applied to each fraction; TP was also assayed on each fraction. AMP was measured from $OD_{260}$ readings. The compounds were eluted in the above sequence; AMP and IP were eluted separately, but the sugar phosphates overlapped.

When using 10 min heating time with the orcinol method, and reading immediately, G1P, G6P, and F6P all gave virtually unmeasurable readings (less than 1% of the readings given by R5P). Using a 20 min heating time increased the sensitivity by 3%, but increased interference from hexose phosphates by 10%. The color was stable; readings after 24 hr at room temperature indicated intermediate sensitivity and specificity. The assay appears to be extremely specific for pentose in the presence of large amounts of hexose. Cysteine-carbazole method was not specific for fructose phosphates, as reported by Bartlett (13), but absorbancy readings for G1P and G6P were about 35% as high as for an equivalent amount of F6P. Only a trace of reaction with cysteine-carbazole was given by R5P.

Another test solution, containing UDPG, FDP, 2PGA, and 3PGA, was chromatographed with a Dowex 1-formate column, which was eluted with 0 to 4 N formate buffer, containing four parts of formic acid and one part of ammonium formate (pH 3.0). UDPG and the two glycerates were eluted simultaneously.

Dowex 1-Chloride Chromatography of Corn Root Tip Extracts

Table 1 shows the levels of TP and IP in pooled trichloroacetic acid extract of 63.2 g of root tips, before and after passage through a Dowex 50 column; recovery of IP was nearly quantitative, while 15% of the TP was retained on the cation exchange resin. Table 2 shows TP and IP levels in several Dowex 50-treated root extracts.

Figs. 1-4 show the results of Dowex 1-chloride chromatography of root tip extracts. The composition of the eluates could not be determined from these data. It appeared from these results that (a) only a crude separation was obtained by the Dowex 1-chloride column (b) the compounds of interest were masked by the presence of various materials giving color formation with TP and sugar phosphate analyses, and others having absorption in the UV spectrum (c) formation of bubbles obscured results in the three sugar assays.

Dowex 1-Formate Chromatography of Eluates from Dowex 1-chloride Separations of Corn Root Tip Extracts

Since Dowex 1-chloride chromatography apparently did not separate sufficiently the phosphorus compounds of interest, and since considerable amounts of unknown materials appeared to mask the results, the solutions eluted from Dowex 1-chloride columns were chromatographed again on Dowex 1-formate columns: each of the five pooled eluates from the chloride columns was passed through a Dowex 1-formate column, and the anions were eluted either with formic acid or a formic acid-ammonium formate buffer.

0.01 N HCl section. Fig. 5 shows the results obtained from the rechromatography of the 0.01 N HCl eluate from a trichloroacetic acid extract of 18.6 g of root tips, using 0 to 1 N formic acid for elution. Similar results were obtained from extracts of 19 to 71 g of root tips.

TABLE 1

PHOSPHORUS CONTENT OF A TRICHLOROACETIC ACID EXTRACT OF 63.2 GRAMS OF CORN ROOT TIPS, BEFORE AND AFTER TREATMENT WITH DOWEX 50

| | P, umoles/g | | % Recovery | |
|---|---|---|---|---|
| | IP | TP | IP | TP |
| Before Dowex 50 | 3.09 | 6.64 | -- | -- |
| After Dowex 50 | 3.03 | 5.63 | 98 | 85 |

TABLE 2

PHOSPHORUS CONTENT OF SEVERAL TRICHLOROACETIC ACID EXTRACTS OF CORN ROOT TIPS, AFTER TREATMENT WITH DOWEX 50

| Wt of root, g | IP, umoles/g | TP, umoles/g |
|---|---|---|
| 57.1 | 4.04 | 7.50 |
| 74.4 | 3.03 | 5.24 |
| 63.2 | 3.03 | 5.63 |
| 29.6 | 2.88 | 7.10 |
| | Av. 3.24 | Av. 6.37 |

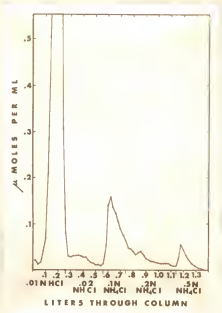


Fig. 1. TP

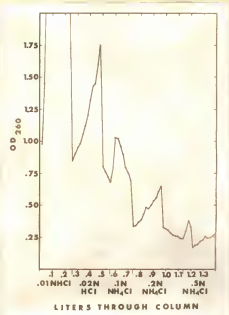


Fig. 2. $OD_{260}$

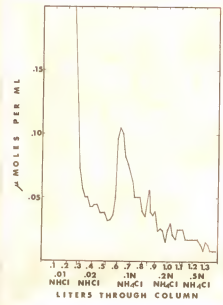


Fig. 3. Anthrone (hexose)


Fig. 4. Cysteine-carbazole (ketose)

Figs. 1-4. DOWEX 1-CHLORIDE CHROMATOGRAPHY OF TRICHLOROACETIC ACID EXTRACTS OF CORN ROOT TIPS. The Dowex 50-treated extracts were run through Dowex 1-chloride columns, and the anions were desorbed by elution with the indicated solutions.

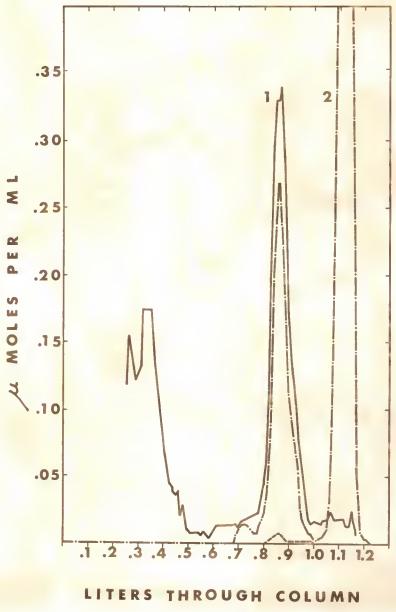


Figure 5. DOWEX 1-FORMATE CHROMATOGRAPHY OF THE WEAK ACID PHOSPHATE COMPOUNDS FROM A TRICHLOROACETIC ACID EXTRACT OF 18.6 GRAMS OF CORN ROOT TIPS. The pooled, neutralized, and diluted 0.01 N HCl section of a Dowex 1-chloride separation was run through a Dowex 1-formate column and the adsorbed anions were eluted with 1.3 l of concave gradient 0 to 1 N formic acid. Assay methods: —·—, TP: ———, Anthrone: ----, Orcinol.

The chromatogram was plotted from TP, anthrone, cysteine-carbazole, and orcinol values. The color formed with the anthrone and cysteine-carbazole reagents was compared with the color formed with fructose standard solutions, and was measured with the Klett colorimeter. The TP was recovered in two well-defined peaks. Table 3 shows the recovery of TP from four root extracts. Linear and concave gradient elutions proved to be equally satisfactory; the only apparent difference was an overall shift of the position of the elution peaks.

A large amount of absorbance in the anthrone method appeared in the first 400-500 ml of eluate. This was probably due to the presence of non-phosphorylated carbohydrates, since no phosphorus was detected in these fractions. The cysteine-carbazole and anthrone assay methods gave similar results when applied to the fractions of Peak 1; addition of the values obtained with those fractions gave 1.46 umoles of keto-hexose/g of roots with anthrone and 1.45 umoles of hexose/g of roots with cysteine-carbazole. The fractions in Peak 2 were essentially negative with anthrone, except for a low "background" absorbance probably due to the persistent elution of non-phosphorylated carbohydrates.

The orcinol method of pentose assay, measured against a R5P stock solution, gave a total of 0.387 umoles of pentose/g of roots, with the peak values at the same elution position as Peak 1. The orcinol result was about what would be expected from the amount of hexose mono-phosphate present, as calculated from anthrone or cysteine-carbazole methods. There is, then, no evidence for the presence of measurable amounts of R5P.

Assay for IP was negative for the fractions in Peak 1; IP assay on the fractions in Peak 2 gave the same values as TP assays (Table 3), indicating that Peak 2 consists of IP.

TABLE 3

TP RECOVERY IN THE DOWEX 1-FORMATE CHROMATOGRAPHY OF THE 0.01 N HCl SECTION FROM DOWEX 1-CHLORIDE CHROMATOGRAPHY OF FOUR CORN ROOT TIP EXTRACTS. (See Fig. 5)

| Weight of roots, g | Peak 1 TP, umoles/g | Peak 2 TP, umoles/g | Total TP, umoles/g | % TP Recovery |
|---|---|---|---|---|
| 63.2 | 0.83 | 2.72 | 3.55 | 94% |
| 22.7 | 0.89 | 3.86 | 4.75 | --- |
| 18.6 | 1.08 | 3.24 | 4.32 | 89% |
| 71.1 | 1.04 | 2.65 | 3.69 | --- |
| | Av. 0.96 | 3.12 | 4.08 | |

Only the first 200-300 ml of eluate absorbed very significantly at 260 mu; no phosphorus was detected in those fractions.

The Peak 1 fractions from an extract of 22.7 g of root tips were tested for ketose by the cysteine-carbazole method using the Beckman DU spectrophotometer, with the following results. Readings were taken on each fraction at 470, 560, 650, and 750 mu (10). Optical density readings were somewhat lower at 650 than at 470 mu, indicating no triose phosphate was present in the fractions tested. There was negligible absorption at 750 mu. Based on the value 0.6 for 0.1 umole of ketohexose (1-cm light path), the ketohexose in Peak 1 was computed to be 0.39 umoles/g. This accounted for 43% of the TP in the Peak 1 fractions. The procedure was repeated with the fractions from another root extract (71.1 g); 40% of the TP was accounted for as ketohexose based on cysteine-carbazole assay. Since the cysteine-carbazole method was found in preliminary experiments to suffer from rather high levels (35%) of interference from G1P and G6P, the absolute values obtained in a mixture did not seem significant in themselves. However, inspection of the ratios of cysteine-carbazole readings/TP content computed for each individual fraction showed the highest values in those fractions corresponding to the descending slope of Peak 1. This is the elution position where F6P has been reported to occur (13), and which was confirmed in these studies.

The pooled fractions of Peak 1 were analyzed enzymatically for G6P; the results indicated the presence of 0.58 umoles of G6P/g of root tips, accounting for 56% of the TP in the peak.

An extract of 38.3 g of roots was tested for G1P by measuring acid-labile (7-min) P in the fractions in Peak 1 (Fig. 6). All

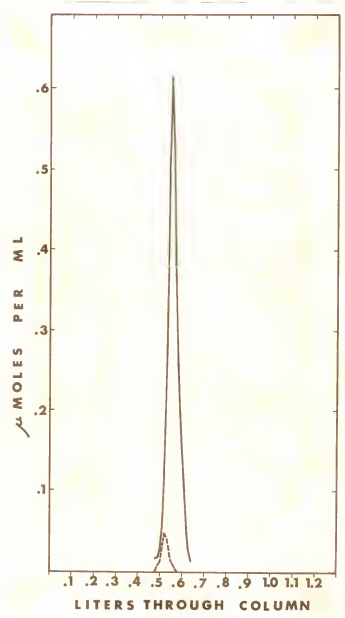


Figure 6. DOWEX 1-FORMATE CHROMATOGRAPHY OF THE SUGAR MONOPHOSPHATES (PEAK 1) FROM AN EXTRACT OF 38.3 GRAMS OF CORN ROOT TIPS. (See Fig. 5 ). Linear gradient. Assay methods: ——— , TP; ----, acid-labile (7 min) P.

fractions were negative for IP; i.e., before hydrolysis. Determination of 7-min P on the same fractions gave peak values in the region of the ascending slope of Peak 1. This is the elution position previously reported for GlP (13), and confirmed in these studies. No other phosphorus compound is known to be eluted in this region, and none of the other sugar phosphates is appreciably labile after 7 min in 1 N HCl at 100 C. Total GlP was computed to be 0.05 umoles/g of root tips.

0.02 N HCl section. Fig. 7 shows the results obtained from the rechromatography of the 0.02 N HCl eluate from a trichloroacetic acid extract of 57.1 g of root tips, using 0 to 1 N ammonium formate for elution. Recovery of P was 60%, about 90% of which was in the two peaks having maximum values at 490 and 720 ml.

$OD_{260}$ and $OD_{290}$ was measured on each fraction. Infinitely large readings were obtained in the first 300 ml at both wavelengths. The UV spectra of these fractions did not resemble those of the nucleotides, and very low levels of P were found in those fractions. Another tall UV peak having the same characteristics, occurred at 770-810 ml.

Three fractions, at 480, 490, and 500 ml, (Fig. 7) were assayed for glycerate by the chromotropic acid method (14). In each case, the spectrum was the same as the characteristic spectrum produced by known MPG under the same test conditions. Further, the ratio of glycerate to TP was about 1:1 for each of the three fractions. From this evidence it was concluded that the three fractions contained essentially pure MPG; the concentration was calculated to be 0.01 umoles of MPG/g of root tips.

The UV spectra of the fractions collected at 720 to 750 ml

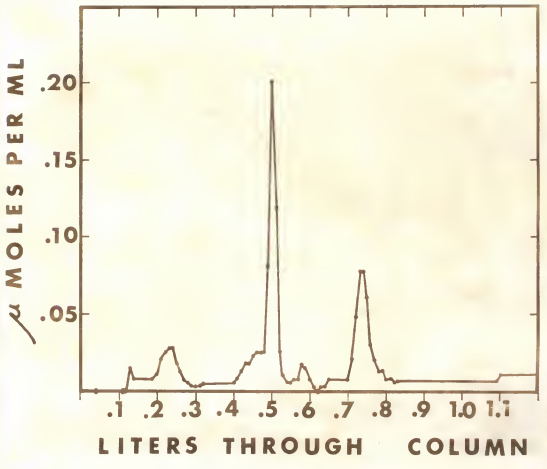


Figure 7. DOWEX 1-FORMATE CHROMATOGRAPHY OF THE 0.02 N HCl SECTION OF A TRICHLOROACETIC ACID EXTRACT OF 57.1 GRAMS OF CORN ROOT TIPS. The pooled, neutralized, and diluted 0.02 N HCl section of a Dowex 1-chloride separation was run through a Dowex 1-formate column, and the adsorbed anions were eluted with 1.2 l of linear gradient 0 to 1 N ammonium formate buffer. Assay: TP.

resembled that of the adenosine phosphates. The absorbancy ratio 250/260 averaged 0.78 for these fractions and the ratio 280/260 averaged 0.16. The pH of the eluates was 6.4. These values were identical to those previously reported for ADP at pH 7.0 (see Table 4). The nucleotide content of the same four fractions was calculated from the molar absorbancy value of the adenosine phosphates, $15.4 \times 10^{-3}$ (61). The ratio of TP to adenine ($OD_{260}$) of the same fractions was 2:1. A Dowex 1-formate chromatogram of an authentic sample of ADP had the peak at the same elution position. From this evidence it was concluded that the compound was ADP; the ADP level was calculated to be 0.85 umoles/g of root tips.

0.1 N $NH_4Cl$, 0.2 N $NH_4Cl$, and 0.5 N $NH_4Cl$ sections. These three eluates from Dowex 1-chloride chromatography were each chromatographed with a formate buffer, using the same techniques described above. In neither instance was there an appreciable recovery of P, most probably due to the presence of relatively high concentrations of chloride ion.

Direct Dowex 1-Formate Chromatography of Corn Root Tip Extracts

Root tip extracts, each representing 50-75 g of tissue, were run through Dowex 1-formate columns after purification with Dowex 50 resin. The adsorbed compounds were eluted with 0 to 4 N ammonium formate buffer, pH 3.0, containing four parts of formic acid and one part of ammonium formate. Linear and concave elution gradients were used. The chromatograms were monitored, as usual, by TP assay on each fraction. Comparison of a concave gradient chromatogram (Fig. 8) with a linear gradient chromatogram (Fig. 10) showed the same sequence of major peaks; but the concave gradient gave better separation of the weakly acidic compounds.

TABLE 4

ABSORBANCY RATIOS AT 250, 260, AND 280 MU

| Compound | $OD_{250/260}$ | | | $OD_{280/260}$ | | |
|---|---|---|---|---|---|---|
| pH | 2 | 7 | 11 | 2 | 7 | 11 |
| ADP* | 0.85 | 0.78 | 0.78 | 0.21 | 0.16 | 0.16 |
| CDP* | 0.46 | 0.83 | 0.83 | 2.07 | 0.98 | 0.98 |
| UDP* | 0.73 | 0.73 | 0.80 | 0.39 | 0.39 | 0.32 |
| UDPG** | 0.76 | 0.76 | 0.82 | 0.38 | 0.39 | 0.34 |
| Peak 6/7*** | 0.83 | 0.79 | 0.83 | 0.47 | 0.45 | 0.43 |

*Pabst Laboratories, Circular OR-7, Ultraviolet absorption spectra of 5'-ribonucleotides, March 1955.

**Sigma, 90%.

***See Figs. 8 and 11.

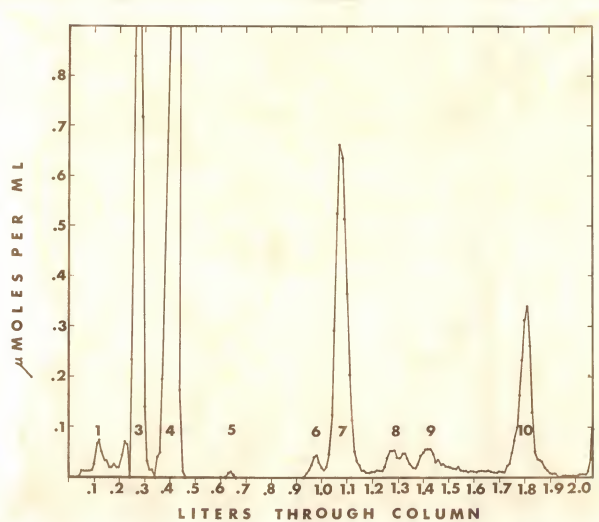


Figure 8. DIRECT DOWEX 1-FORMATE CHROMATOGRAPHY OF A TRICHLOROACETIC EXTRACT OF 51.8 GRAMS OF CORN ROOT TIPS. The extract, after purification with Dowex 50 (hydrogen form), was passed through a Dowex 1-formate column, and eluted with 2.1 l of concave gradient 0 to 4 M formate buffer containing four parts of formic acid and one part of ammonium formate (pH 3.0). Assay: TP.

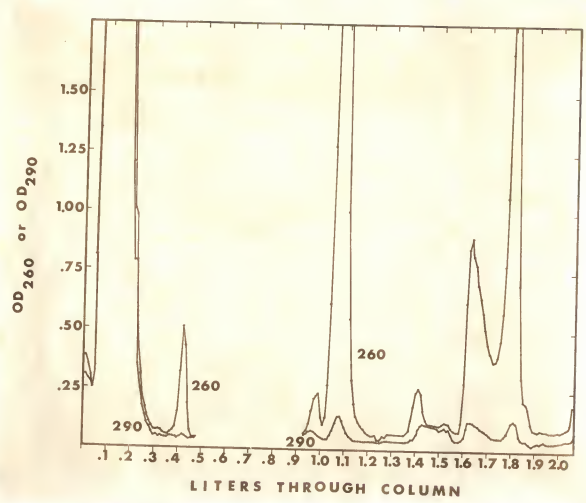


Figure 9. DIRECT DOWEX 1-FORMATE CHROMATOGRAPHY OF A TRICHLOROACETIC ACID EXTRACT OF 51.8 GRAMS OF CORN ROOT TIPS. (See Fig. 8). Assay: $OD_{260}$ and $OD_{290}$, as indicated.

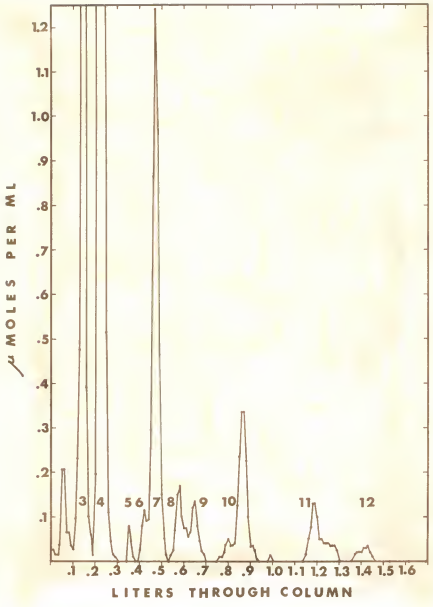


Figure 10. DIRECT DOWEX 1-FORMATE CHROMATOGRAPHY OF A TRICHLORO-ACETIC ACID EXTRACT OF 71.9 GRAMS OF CORN ROOT TIPS. The procedure followed was described in Fig. 8; linear gradient elution. Assay: TP.

In a typical experiment (Fig. 8), using 51.8 g of root tips, 6.75 umoles of P/g of root tips were added to the column, and 5.52 umoles (82%) were recovered in the individual fractions. Of the P recovered, from averages of several runs, 4.92 umoles (87%) appeared in the four largest peaks, as follows: Peak 3, 0.93 umoles/g; Peak 4, 3.61 umoles/g; Peak 7, 0.70 umoles/g; Peak 10, 0.25 umoles/g. Peak 3 corresponded to the hexose monophosphate peak (Peak 1) in Dowex 1-formate chromatography of the 0.01 N HCl Dowex 1-chloride eluate (Table 3). Peak 4 was IP, corresponding to Peak 2 in the same table.

The fractions of Peak 3 were pooled, freed of ammonium and formate ions, and analyzed enzymatically for F6P. Analysis showed the presence of 0.15 umoles of F6P/g of root tips: this accounted for 14% of the TP in Peak 3.

Fig. 9 shows $OD_{260}$ and $OD_{290}$ measurements of the eluates from concave gradient chromatography of 51.8 g of root tips. Extremely high levels of absorbancy at both wavelengths were found in the first 300 ml, indicating the presence of large amounts of unknown materials absorbing in the near UV spectrum.

A UV peak, possibly representing a nucleotide, was found at 390-450 ml. The position of the peak did not correspond exactly to Peak 4 (Fig. 8). Using the molar absorbancy value $15.4 \times 10^{-3}$, the total UV-absorbing material would be less than 0.02 umole/g, or 0.7% of the phosphorus in Peak 4, on a monophosphate basis. The identity of the compound was not determined.

A small UV-absorbing peak was found at 950-1000 ml; it was not identified. Another small peak at 1380-1440 ml was not identified. A fairly large peak at 1600-1700 ml did not correspond to the TP

chromatogram, and its identity was not ascertained. Two peaks were found at 1030-1130 ml and 1750-1840 ml.

The fractions making up each peak in Dowex 1-formate direct chromatography were pooled, ammonium and formate ions were removed, and UV absorbancy was measured on each combined peak (Fig. 11). Only Peak 6/7 (combined) showed a UV absorption spectrum similar to a nucleotide. Peak 6 contained 4% of the TP in the combined peak 6/7. The $OD_{max}$ of Peak 6/7 was 262 mu, and the complete UV absorption spectra at pH 2, 7, and 11 (not shown) were similar to spectra of the uridine nucleotide, and unlike the spectra of the other nucleotides. The absorbancy ratios 250/260 and 280/260, given in Table 4, indicated that Peak 6/7 probably contained UDP or UDPG, or both. Enzymatic assay of the peak indicated the presence of 0.13 umoles of UDPG/g of root tips, corresponding to 0.26 umoles P/g root tips; 38% of the TP in Peak 6/7 can be accounted for as UDPG.

Orcinol, anthrone, chromotropic acid, cysteine-carbazole, and acid-labile (7-min) P assay methods were applied to each of the pooled peaks. No clearcut results were obtained, because the peaks all contained impurities which interfered with colorimetric assays.

ATP Assay of Corn Root Tip Extracts

The firefly lantern extract was used to measure the ATP level in trichloroacetic acid extracts of root tips. The results indicated 0.15 umoles of ATP/g of root tips in the acid-free extract. When the extract, containing trichloroacetic acid, was analyzed after neutralization with 0.1 M Tris buffer to pH 7.4, the apparent ATP concentration was only 20% of that observed in the acid-free extract, possibly due to a salt effect. ATP content of a perchloric acid extract of a single root tip was 0.18 umoles/g.

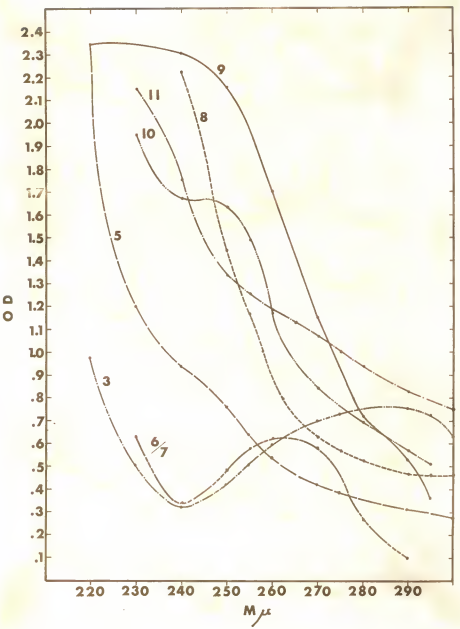


Figure 11. ULTRAVIOLET SPECTRA OF THE POOLED TP PEAKS FROM DIRECT DOWEX 1-FORMATE CHROMATOGRAPHY OF TRICHLOROACETIC ACID EXTRACTS OF CORN ROOTS. The fractions of each numbered peak (see Figs. 8 and 10) were pooled, ammonium ion was removed with Dowex 50 (hydrogen form), formic acid was removed by extracting with ether; each solution was neutralized before its UV absorbancy was measured.

Phosphorus Compounds Identified in the Root of the Etiolated Corn Seedling

Table 5 shows the concentrations of the P compounds found in the seedling root. The values for MPG and ADP are, undoubtedly, much too low, because about 40% of the TP failed to adsorb to the Dowex 1-formate column.

TABLE 5

PHOSPHATE COMPOUNDS OF CORN ROOT TIPS

| Compound | umoles/g of corn root tips |
|---|---|
| IP* | 2.65-3.86 |
| G1P | .05 |
| G6P | .58 |
| F6P | .15 |
| UDPG | .13 |
| ADP | .85 |
| ATP | .15 |
| MPG | .01 |

*Range of values from four extracts. Each other value from a single extract.

## DISCUSSION

Previous workers have encountered difficulties in the separation and analysis of the phosphorus compounds in plant tissues, and one of these, Albaum ( 3 ), declared the use of ion exchange chromatography for this purpose "not feasible," because of the swamping of the analyses at every step by large quantities of phosphorus compounds of various compositions.

Considerable difficulty was also encountered in these studies for similar reasons. The corn seedling root tissue contained high levels of phosphorus compounds of unknown composition, which were difficult to separate from the glycolytic intermediates and nucleotides which were of primary interest. However, the results of the investigations reported in this paper indicate that anion exchange methods show promise for the separation and quantitative analysis of those compounds of corn seedling roots, and it is believed that the further application of these methods will result in the analysis of many others.

It was concluded that the use of colorimetric methods for the assay of sugar phosphates in mixtures is of limited value in such work. An exception was the orcinol assay method for pentose. The phosphorus compounds were not separated sufficiently on Dowex 1-chloride columns to analyze them quantitatively from TP assay, UV absorbancy measurements, and colorimetric sugar analyses on the

eluted fractions. The eluates from Dowex 1-chloride columns could be rechromatographed on finer mesh Dowex 1-formate columns, however, resulting in further separation of phosphorus compounds. The compounds thus separated appeared to be in almost pure form, except for the hexose monophosphates, which were not separated from each other under the test conditions. This method of rechromatography of chloride-containing eluates was unfortunately limited in its usefulness to eluates 0.02 N or less in chloride ion, because of the swamping of the phosphorus compounds at high chloride concentrations. Slower elution of the Dowex 1-chloride column with smaller amounts of chloride eluent may overcome this difficulty.

The use of Dowex 1-formate columns to separate directly the tissue extracts, without prior separation on Dowex 1-chloride offers promise. The ammonium and formate ions, unlike the chloride ion, can be essentially removed from the eluates. Dowex 1-formate rechromatography of selected portions of these eluates, using various ammonium formate buffers, would be expected to provide further separation.

The enzymatic analyses proved to be useful when dealing with eluates of varying degree of purity. It may prove practical, as well as expedient, to perform such analyses on the five relatively mixed eluates from Dowex 1-chloride columns. The firefly lantern extract (luciferase) method was found to be an extremely sensitive method for measuring ATP in plant tissue.

The concentrations of the phosphorus compounds identified in acid extracts of corn roots should be confirmed by additional tests.

## SUMMARY

Methods were described for the separation and analysis of glycolytic intermediates in etiolated corn seedling roots, with the use of ion exchange column chromatography. The following compounds were identified: inorganic phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, monophosphoglyceric acid, adenosine diphosphate, adenosine triphosphate, and uridine diphosphate glucose.

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## BIOGRAPHICAL SKETCH

Byron H. Wise was born 12 February 1925 in Gainesville, Floride. He graduated from P. K. Yonge Laboratory School in 1942.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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